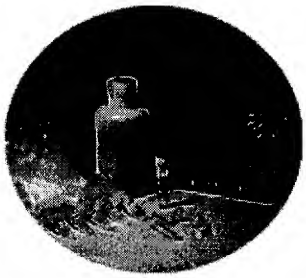
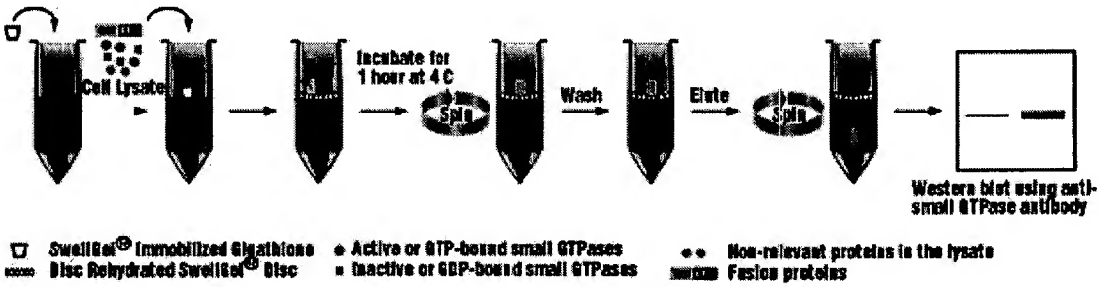
EZ-Detect Ras, Cdc42, Rac1 and Rho Activation Kits



Monomeric p21 GTP-binding proteins (small GTPases) serve as molecular switches in regulating a wide range of essential biochemical pathways in eukaryotic cells. The families of Ras and Rho (including Cdc42, Rac1 and Rho) are of special interest as they influence the cell's response to the changing environment. Research results have indicated that these proteins regulate numerous cell functions such as proliferation, differentiation, transformation, apoptosis, migration, actin cytoskeleton reorganization, and cell cycle progression.[1] Like other G-proteins, small GTPases cycle between an inactive, GDP-bound state and an active, GTP-bound state.

The new Pierce EZ-Detect Rac1, Cdc42, Ras and Rho Activation Kits measure the activation of small GTPases by isolating them via their specific downstream effectors.[1-6] The respective binding domain of the downstream effector for each small GTPase is expressed as a GST-fusion protein which, when immobilized on a resin, is used to pull down the active or GTP-bound GTPase (Figure 1). GST-RBD (Ras binding domain) of Raf1 can pull down active Ras,[2] GST-PBD (p21 binding domain) of Pak1 can pull down active Rac1 and Cdc42,[3,4] and GST-Rhotekin-RBD can pull down active Rho.[5-7] The pulled-down active GTPase is then detected by a Western blot using a specific antibody.



One general characteristic of pull-down assays is the small volume of resin required for each sample. The EZ-Detect Small GTPase Activation Kits have eliminated the inconsistencies associated with measuring small volumes of resin slurry by supplying the immobilized glutathione resin in the SwellGel Disc format.[8]

Each EZ-Detect Small GTPase Activation Kit includes GST-PBD or GST-RBD, SwellGel Immobilized Glutathione Discs (equivalent to 50 µl resin), lysis/binding/washing buffer, primary antibody, GTPγS, GDP, 2X SDS sample buffer, spin columns and collection tubes. Each kit contains enough components for 30 pull-down assays.

Methods

NIH3T3 cells were grown to 100% confluency in 100 mm culture dishes and lysed in 500 µl lysis/binding/washing buffer. The clarified cell lysate (500 µg) was treated with 0.1 mM GTPγS or 1.0 mM GDP in the presence of 10 mM EDTA, pH 8.0 at 30°C for 15 minutes (to activate or inactivate Rac1, Cdc42 and Ras) or 30 minutes (to activate or inactivate Rho).

The nucleotide exchange reaction was terminated by adding $MgCl_2$ and placing samples on ice.[3,4] The treated lysates (500 µg) were incubated with GST-Raf1-RBD (to pull down active Ras), GST-Pak1-PBD (to pull down active Cdc42 or Rac1) or GST-Rhotekin-RBD (to pull down active Rho) in the presence of SwellGel Immobilized Glutathione at 4°C for 1 hour in a spin column. After incubation, the mixture was centrifuged at 8,000 x g to remove the unbound proteins. The resins were washed three times with lysis/ binding/wash buffer and the sample was eluted by adding 50 µl of 2X SDS sample buffer and boiling at 95°C for 5 minutes. Half (25 µl) of the sample volumes were analyzed by SDS-PAGE (4-20% polyacrylamide mini-gel) and transferred to a nitrocellulose membrane. The active Ras, Cdc42, Rac1 or Rho was detected by Western blotting using a specific antibody. ImmunoPure Goat anti-Mouse Antibody (H+L) conjugated with horseradish peroxidase (1:100,000 dilution) (Product # 31430) was used as the secondary antibody. Detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Product # 34079) followed by exposure to X-ray film (average exposure time was 10-30 seconds).

Discussion

To determine the specificity and function of GST-Raf1-RBD, GST-Pak1-PBD or GST-Rhotekin-RBD, cell lysate was treated with GTPγS to activate the endogenous Ras, Cdc42, Rac1 or Rho or treated with GDP to inactivate these small GTPases.[3,4] A strong signal for Rac1, Cdc42, Ras or Rho was detected by Western blotting using GTPγS-treated cell lysate, while very little or no signal was detected when GDP-treated cell lysate was used (Figure 2). Furthermore, no signal was detected when GST alone was incubated with GTPγS-treated cell lysate (negative control; data not shown). Therefore, the EZ-Detect Ras, Cdc42, Rac1 or Rho Activation Kit can specifically monitor each active small GTPase.

Figure 2. Detection of the active Ras, Cdc42, Rac1 and Rho using EZ-Detect Ras, Cdc42, Rac1 and Rho Activation Kits. NIH3T3 cell lysate treated with GTPγS or GDP was incubated with GST-Raf1-RBD, GST-Pak1-PBD or GST-Rhotekin-RBD and SwellGel Immobilized Glutathione. Half of the eluted pull-down samples (25 µl) and 20 µg of lysate were analyzed by Western blotting using anti-Rac1, anti-Cdc42, anti-Pan-Ras or anti-Rho antibody. There were no active GTPases detected (see GDP lanes).

To determine the sensitivity of the EZ-Detect Small GTPase Activation Kits, different amounts of GTPγS-treated cell lysate were incubated with GST-Pak1-PBD, followed by Western blot analysis using anti-Rac1 antibody (Figure 3). Active Rac1 was detected using as little as 45 µg of lysate in the pull-down assay.

Figure 3. Sensitivity of EZ-Detect Rac1 Activation Kits. Different amounts of NIH3T3 cell lysate treated with GTPγS were incubated with GST-Pak1-PBD and SwellGel Immobilized Glutathione. A GDP-treated cell lysate (500 µg) was incubated with GST-Pak1-PBD and SwellGel Immobilized Glutathione as a negative control. Half of the eluted pull-down samples (25 µl) and 20 µg of lysate were analyzed by Western blotting using anti-Rac1 mouse monoclonal antibody.

The EZ-Detect Ras, Cdc42, Rac1 and Rho Activation Kits are also highly efficient in the pull-down of active small GTPases from multiple samples that were prepared following different physiological treatments. Serum-starved NIH3T3 cells were stimulated by 10% serum for various times up to 1 hour. Cell lysates (300 µg) were prepared at the indicated times (Figure 4) and incubated with GST-Raf1-RBD and SwellGel Immobilized Glutathione. Biphasic activation of Ras was detected at 1 minute and 30-60 minutes in response to serum. Because the EZ-Detect Small GTPase Activation Kits provide a simple and convenient protocol, multiple samples can be easily processed simultaneously. Furthermore, the sensitivity of the system allows monitoring and measurement of various active states of small GTPases.[2,9]

Figure 4. Detection of active Ras stimulated by serum using EZ-Detect Ras Activation Kits. NIH3T3 cells were serum-starved for 24 hours, followed by treatment with 10% FBS at the indicated time. The lysate (300 µg) was incubated with GST-Raf1-RBD and SwellGel Immobilized Glutathione. Half of the eluted pull-down samples (25 µl) and 20 µg of lysate were analyzed by Western blotting using anti-Pan-Ras mouse monoclonal antibody.

The EZ-Detect Ras, Cdc42 and Rho Activation Kits were compared to competitor kits. To measure active Ras, 500 µg of GTPγS-treated cell lysate was incubated with the respective GST-Raf1-RBD from each kit. Results showed a greater signal from the EZ-Detect Kit when compared to Competitor U's kit. The performance of the EZ-Detect Cdc42 Activation Kit was similar to Competitor U's kit, but it was more sensitive than Competitor C's kit. The EZ-Detect Rho Activation Kit showed greater sensitivity in detecting active Rho from cell lysate than did Competitor U's kit.

Conclusions The results of this study show that the Pierce EZ-Detect Ras, Cdc42, Rac1 and Rho Activation Kits are simple, convenient and powerful tools in measuring active small GTPases.

Figure 5. Performance comparison of EZ-Detect Ras, Cdc42 and Rho Activation Kits with competitor kits. To pull down active Ras, NIH3T3 cell lysate (500 µg) was treated with GTPγS and incubated GST-Raf1-RBD from Pierce or from a competitor. For pull-down of active Cdc42 or active Rho, the GTPγS-treated NIH3T3 cell lysate was incubated with GST-Pak1-PBD or GST-Rhotekin-RBD from Pierce or competitors (according to the manufacturer's protocol). Half of the eluted pull-down samples (25 µl) were analyzed by Western blotting using anti-Pan-Ras, anti-Cdc42 or anti-Rho antibody.

Features

- High sensitivity – SwellGel Technology combined with enhanced SuperSignal Technology allows the use of less sample
- Convenience – no need to express and purify GST-PBD or RBD fusion proteins
- Speed – simultaneous incubation of cell lysate with GST-PBD or RBD and the pre-measured SwellGel Immobilized Glutathione Disc directly in a spin column
- Ease-of-use – pull-down conditions have been optimized for immediate success, even for first-time users
- Efficiency – the spin column and receiver tubes allow efficient separation of liquid and resin, preventing sample loss

References

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Ordering Information

Instruction Book MSDS

Buy	Product #	Description	Pkg. Size	Files	Price
Add	89854	EZ-Detect Rho Activation Kit	kit		$550.00
Add	89855	EZ-Detect Ras Activation Kit	Kit		$550.00
Add	89856	EZ-Detect Rac-1 Activation Kit	Kit		$550.00
Add	89857	EZ-Detect CDC42 Activation Kit	Kit		$550.00

Rhotekin-RBD Protein GST Beads Cat. # (RT02)

Material
The Rho-GTP binding domain (RBD) of the mouse rhotekin protein has been overexpressed as a GST-tagged recombinant protein in E. coli. The Rhotekin-RBD protein (RT02) is 95% pure (see Figure A). Each tube of RT02 contains 500 ug of bead bound protein.

Fig.A Rhotekin-RBD GST tagged protein Purity Detemination

Fig. A. Lane 1 shows a 10 ug sample of recombinant Rhotekin-RBD GST tagged protein (molecular weight approx. 35kD). The protein was separated by electrophoresis in a 4-20% SDS-PAGE system. Protein was viewed by staining with coomassie blue. Protein quantitation was performed using the Advanced Protein Assay (cat# ADV01).

Biological activity

The Rhotekin-RBD protein specifically recognizes and binds to the active "GTP-bound" form of Rho protein. It has a much lower affinity for the inactive "GDP-bound" form of Rho. Biological activity of Rho-PBD protein is therefore determined by its selectivity for GTP-Rho protein.

Catalogue number: RT02

Price ($): Per Kit = $250

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REFERENCES:
Ren, X.-D. and M.A. Schwartz, "Determination of GTP-loading on Rho," Meth. Enzymol. 325: 264, 2000.
Xiang-Dong Ren, et al. EMBO J. 18: 578-585, 1999.
Application References...

PURITY: >90%

SOURCE: GST-tagged fusion protein corresponding to residues 7-89 of mouse Rhotekin Rho Binding Domain, expressed in E. coli

STABILITY: 6 months at -70C

APPLICATIONS:
Affinity Precipitation: 30 mcg